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TITLE: Evaluation of DNA Methylation as a Target for Intraductal Therapy for Ductal Carcinoma in Situ of the Breast

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14. ABSTRACT

In ductal carcinoma in situ(DCIS), the malignant cells are confined within the basement membrane, and so an ideal candidate for local therapies. Because DNA methylation is a potentially reversible mechanism for tumor suppressor gene inactivation, it is an intriguing target for molecular therapeutics. In this study we have documented significant methylation in eight tumor suppressor genes in DCIS. We have successfully performed ductal lavage in 24/27 patients undergoing surgery for DCIS without any complications. Unfortunately, we were able to successfully identify and lavage the malignant duct in only 25% of cases, half of which were identified because the patient presented with discharge. In the absence of nipple discharge, only 14% of the lavaged ducts were the malignant duct. While these data do not rule out the potential of targeting DNA methylation for intraductal therapy for DCIS, we need better methods for identifying the malignant ductal orifices before proceeding to clinical trials of intraductal therapy.

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Ductal Carcinoma in situ of the Breast, intraductal therapy, ductal lavage, DNA methylation

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Introduction: Ductal carcinoma *in situ*(DCIS), the preinvasive form of infiltrating ductal carcinoma of the breast, currently accounts for 20-30% of breast cancers and is treated by surgically removing the involved ducts. In DCIS, the malignant cells have not having invaded through the basement membrane and therefore have not gained access to the lymphatics or the systemic circulation. DCIS is a local disease, and so an ideal candidate for local therapies. DNA methylation is one mechanism for tumor suppressor gene inactivation. It is an early event in the course of malignant progression in several tumor systems. Because methylation is a potentially reversible mechanism for tumor suppressor gene inactivation, it is an intriguing target for molecular therapeutics. Drugs, such as 5-aza-deoxycytidine (DAC), are available that can reverse methylation changes and prevent tumor suppressor gene-related neoplasia *in vivo*. **Hypothesis: DNA Methylation is altered in DCIS and is a therapeutic target for intraductal therapy. Specific Aim 1:** To document the methylation status of a panel of tumor suppressor genes in DCIS. **Specific Aim 2:** Document the feasibility of an intraductal approach to DCIS. **Specific Aim 3:** Identify a dose or range of doses of DAC with biologic activity and acceptable side effects when delivered intraductally to patients with DCIS (Phase I trial).

Body: Due to significant administrative delays, final approval by your HSRRB was granted on 4/14/05. As a result this report reflects work performed in the between 4/15/05 and 12/31/06.

Task 1: Document the feasibility of an intraductal approach to DCIS (Months 1-24)

a) Perform Ductal Lavage (DL) on 50 patients with core-biopsy proven DCIS (Months 1-20)

Between 4/15/05 and 7/15/07, 27 patients with core biopsy proven DCIS were enrolled and ductal lavage was successfully completed in 24. There have been no complications to date. In 2 patients we were unable to elicit nipple aspirate fluid and were unable to identify a duct for ductal lavage. One patient withdrew from the study prior to surgery. 8 patients underwent mastectomy and 16 patients underwent breast conserving surgery. Lavage specimens were classified by the cellularity of the specimen: acellular (no ductal epithelial cells seen), low (<10 epithelial cells per 20X field), moderate (10-30 epithelial cells per 20X field), and high (>30 epithelial cells per 20X field). Three patients presented with nipple discharge, the remainder had abnormalities found on routine screening mammograms. All 3 patients with nipple discharge had DL specimens of high cellularity. In 3 patients there was no residual DCIS in the surgical (Surg) specimen (all DCIS had been removed on core biopsy). Each of these 3 cases had DL specimens of low cellularity. DL and Surg specimens were scored for 12 cytological criteria: epithelial cell size, nuclear size, cell arrangement, microcalcifications, anisonucleosis, nuclear membrane irregularity, chromatin, nucleoli, mitoses, multinucleation, necrotic debris and nuclear/cytoplasmic ratio. The scores for each feature were added to generate a total cytologic score ranging from 12 (completely benign features) to 33 (highly malignant features). Each specimen was scored in duplicate (at 2 different sittings) by a dedicated cytopathologist and the average of the 2 scores was calculated. Cells were felt to be benign if the score was between 12 and 16, atypical if between 16 and 24, and malignant if between 25 and 33. Correlation was defined as good if both the DL and Surg scores were in the same cytologic class, minimal if in adjacent classes, and poor if one was benign and the other malignant.

There was a definite learning curve. For the first 5 ductal lavage (DL) procedures, any fluid producing duct was lavaged. Of these 5, 2 were acellular and 2 were of low cellularity. There was no more than minimal correlation between the DL cytology scores and the surgical specimen cytology scores. After seeing these results, we made an effort to only cannulate ducts that were in close to the same anatomic position on the nipple as the DCIS is in the breast based on mammography (ie 9:00 peripheral duct for a 9:00 peripheral lesion). Of the next 19 DL specimens, only 2 were acellular (10.5%), 5 were of low cellularity (26.3%), and 6 each were of moderate and high cellularity (31.6%). Of note, of the 6 cases with high cellularity, 3 (50%) were patients who

presented with nipple discharge. Only 3/21 (14%) patients without discharge had DL specimens with high cellularity.

b) Compare the cytology of the ductal lavage specimen to the histology of the surgical specimen (Months 6-24)

Of the 20 evaluable DL specimens, 6 (30%) scored in the benign range, 8 (40%) scored in the atypical range and 6 (30%) scored in the malignant range. Of the 21 evaluable surgical specimens, 2 low grade DCIS (9.5%) scored in the atypical range, with the remaining 19 (90.5%) scoring in the malignant range. Table 1 shows the summary data on the cytologic scoring for the 24 samples.

Table 1: Cytologic Scores for DL and Surg specimens by specimen cellularity

Cellularity	n (%)	Average DL Score	Average Surg Score	P Value DL vs Surg
Acellular	4 (17)	NA	26.6	
Low	7 (29)	14.4	27.4*	<0.0001
Moderate	7 (29)	21.1**	28.7	0.008
High	6 (25)	28.5***	30.1	0.35
All	24	21.0	28.5	<0.0001

* 3 cases had no residual cancer in the surgical specimen
** p=0.02 compared to paucicellular
***p=0.02 compared to moderate

DL cytologic score increased significantly with increasing cellularity of the specimen, suggesting that the more abnormal the epithelial cells, the more cells can be harvested by lavage. DL specimens of low and moderate cellularity had significantly lower cytologic scores than seen in the Surg specimens, suggesting that the DL did not retrieve malignant cells (the malignant duct was not cannulated). Cytology scores of DL specimens with low cellularity were in the benign range, those with moderate cellularity were in the atypical range, and those with high cellularity were in the malignant range.

Cytologic scores for both the surgical and DL specimens were available for 17 of the 24 patients (70.8%, 4 patients had acellular DL specimens and 3 had no residual cancer in the surgical specimens). Correlation was classified as poor, minimal, or good as defined above. Table 2 shows the cytologic correlation for the 17 cases.

Table 2: Correlation between DL and Surg specimen cytology

Cellularity (n)	Poor	Minimal	Good
Low (4)	2	2	0
Moderate (7)	2	4	1
High (6)	0	1	5

p = 0.036

Cytologic correlation was significantly associated with high cellularity of the DL specimen. Of the 6 cases with good cytologic correlation, 3 (50%) presented with nipple discharge. Two of 6 patients with good correlation had lavage of a dry (non-fluid producing) duct.

c) Assess the distribution of intraductally injected ink in the surgically resected pathology specimens (Months 6-24)

The distribution of the intraductally injected ink was defined as absent (no ink seen in any ducts), away from DCIS (ink seen in ducts that were distant from the malignant ducts), adjacent to DCIS (ink seen in uninvolved ducts adjacent to malignant ducts), or in DCIS (ink identified within the malignant ducts). By definition, and intraductal ink seen in uninvolved ducts in lumpectomy specimens were adjacent to DCIS. In 5 cases, no ink was seen. One of these cases was a mastectomy specimen and all of the dye had extravasated into the retroareolar space (the first patient done). In the remaining 5 mastectomy specimens, 2 showed ink in ducts distant from the malignant ducts, 2 had ink in the malignant ducts, and 1 had ink in adjacent ducts. Of the 16 lumpectomy specimens, 4 had no ink identified in the specimen, suggesting that the ink had been injected into an unresected ductal system. Of note, none of these patients had any adverse effects, confirming that India ink is a biologically inert substance and well tolerated when injected intraductally. Table 3 shows the ink distribution patterns for the 24 surgical specimens.

Table 3: Ink distribution patterns in surgical specimens by cellularity of the associated DL specimen

DL Cellularity	Absent	Away from DCIS*	Adjacent to DCIS	In DCIS Ducts*
Acellular, n=4	2	2	0	0
Low, n=7	2	0	5	0
Moderate, n=7	1	0	5	1
High, n=6	0	0	1	5
All, n=24	5 (21%)	2 (8%)	11 (46%)	6 (25%)

* Only seen when DL specimen has good cytologic correlation with surgical specimen
** Only visualized in mastectomy specimens

The ink distribution confirms the findings suggested by the cytology data: Malignant ducts were successfully cannulated and lavaged in only 25% of patients, half of whom presented with nipple discharge. The remaining DLs accessed uninvolved ducts either adjacent to the cancer (46%) or distant (29%).

The data from Task 1 demonstrate that DL can be performed successfully in women undergoing surgery for DCIS and that intraductal injection of India ink is well tolerated. Unfortunately, in the absence of nipple discharge the malignant duct is accessed in only 14% of patients. The presence of nipple aspirate fluid (NAF) did not predict a malignant duct. Ductal identification based on anatomic location was more effective at identifying the malignant ducts (6/19 cases, 32%) than simply the presence of NAF (0/5). While these data confirm the feasibility of an intraductal approach to DCIS, better methods of duct selection are needed before proceeding to clinical trials.

Task 2: Document the methylation status of a panel of tumor suppressor genes in DCIS (Months 6-24)

Based on data generated in another study, eight of the panel of 20 tumor suppressor genes originally planned to be studied (APC, CALCA, cyclinD2, GSTP1, HPP1, MyoD1, RARB2, and RASSF1) were found to be hypermethylated in invasive breast cancers compared to normal breast tissues (unpublished data). Based on these findings, we documented (in another study) the methylation status of these 8 genes in 50 archival surgical specimens from patients with biopsy proven DCIS and found significant levels of methylation in all eight genes (Table 4).

Table 4: % of Samples Methylated

Gene	APC	CALCA	cyclinD2	GSTP1	HPP1	MYOD1	RARβ2	RASSF1
DCIS	57	26	61	22	39	65	78	91

We focused on these eight genes in the DL and surgical specimens.

a) Perform methylation analyses on the surgical specimens from 50 patients with core biopsy-proven DCIS (Months 2-24), and b)Perform methylation analyses on the ductal lavage specimens from 50 patients with core biopsy proven DCIS (Months 6-24)

Of the 24 DL samples, 11 were either acellular or of low cellularity. These 11 samples did not yield sufficient DNA for methylation analyses. The summary methylation data for the remaining 13 samples is shown in Table 5.

Table 5: Methylation of Tumor Suppressor genes in DL and Surgical specimens.

			% of Samples Methylated							
Cytologic Correlation	**Spec Type**	**n**	**APC**	**CALCA**	**cyclinD2**	**GSTP1**	**HPP1**	**MYOD1**	**RARβ2**	**RASSF1**
Poor	**DL**	2	0	0	0	0	0	0	50	50
	Surg	2	100	50	50	0	50	50	100	100
Minimal	**DL**	5	60	40	40	0	20	20	0	100
	Surg	5	60	60	80	60*	80	40	60*	100
Good	**DL**	6	33	50	50	17	17	50	50	83
	Surg	6	17	50	33	17	0	50	33	83
All	**DL**	13	38	38	38	8	15	31	31	85
	Surg	13	46	54	54	31	38	46	54	92

*$p<0.05$ compared to DL specimens

While DNA methylation was identifiable in all DL specimens, methylation seemed to be related to the extent of cytologic abnormality. The closest correlation between the methylation seen in the DL and that seen in the Surg specimen was seen in those samples with good cytologic correlation. The presence of DNA methylation did not prove the presence of malignant cells. DNA methylation is a common finding in DCIS and may be an appropriate target for intraductal therapies.

Task 3: Identify a dose or range of doses of intraductal DAC with biologic activity and acceptable side effects (Phase I trial)(Months 25-48).

This task was not performed. Due to the results from task 1, proceeding to a clinical trial of intraductal therapy is not indicated at this time, as we have not been able to reliably cannulate the malignant ducts.

Key Research Accomplishments:

- Successfully performed DL in 24 women undergoing surgery for DCIS
- Evaluated the correlation between the cytology of the DL specimen and that of the surgically resected DCIS
- Evaluated the DNA methylation patterns in the DL specimens compared to those in the surgically resected DCIS

Reportable Outcomes: Manuscript in preparation based on the data from Task 1.

Data on DNA methylation in DCIS presented at the AACR and abstract published (listed in References, below).

Conclusions:

- DL can be performed successfully in women undergoing surgery for DCIS
- Itraductal injection of India ink is well tolerated.
- I the absence of nipple discharge, the malignant duct is accessed in only 14% of patients.
- The presence of nipple aspirate fluid (NAF) did not predict a malignant duct.
- Ductal identification based on anatomic location was more effective at identifying the malignant ducts (6/19 cases, 32%) than simply the presence of NAF (0/5).
- These data confirm the feasibility of an intraductal approach to DCIS, better methods of duct selection are needed before proceeding to clinical trials.
- DNA methylation was identifiable in all DL specimens, methylation seemed to be related to the extent of cytologic abnormality.
- DNA methylation is a common finding in DCIS and may be an appropriate target for intraductal therapies.

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Zapparoli, GV, Kim, SJ, Kachel, CA, Chiriboga, L , Sullivan, RF, **Skinner, KA**. "Methylation Patterns in Preinvasive Breast Cancer" *Proceedings of the AACR*, Volume 47, 2006

Appendices: N/A